

Combined phosphoflow with RNA flow cytometry

CM Claire Ma JM John C Marioni AR Arianne C Richard GG Gillian M Griffiths

Updated date: Jan 25, 2021

 An abbreviated version of this protocol was published in eLIFE in May 2020

Stimulation strength controls the rate of initiation but not the molecular organisation of TCR-induced signalling

DOI: 10.7554/eLife.53948

Detailed protocol

Combined PrimeFlow and Phosphoflow (Claire Ma, Griffiths lab 2020)

Day 1: Antibody Staining, fixation and permeabilization

1. Homogenize mouse spleen.
2. Perform CD8⁺ T-cell isolation using the MACS Miltenyi kit:
 - Resuspend the cell pellet in 40µL of MACS buffer per 10⁷ total cells.
 - Add 10µL of Biotin-Antibody Cocktail per 10⁷ total cells.
 - Mix well and incubate for 5min in the fridge (2-8°C).
 - Add 30µL of MACS buffer per 10⁷ total cells.
 - Add 20µL of Anti-Biotin Microbeads per 10⁷ total cells.
 - Place LS Column in the magnetic field of a MACS separator.
 - Prepare column by rinsing with 3ml of MACS buffer.
 - Apply cell suspension onto the column and collect flow through containing unlabelled cells, representing the enriched CD8⁺ T cells.
 - Wash column 3 times with 3ml of MACS buffer, collect and pellet unlabelled cells.
3. Resuspend cells in cold serum-free RPMI.
4. Stain with Fixable Viability Dye in serum-free RPMI at room temperature for 10min in the dark.
5. Wash cells in cell culture media (at least 10% FBS), pellet and resuspend in culture media.
6. Perform stimulation for desired time points, aiming for at least 2*10⁶ cells per condition.
7. Move stimulation plate to ice, then transfer cells to FACs tubes, keeping tubes on ice.
8. Spin 500 x g 5min at 4°C. Discard supernatant.
9. Wash with cold PBS, spin 500 x g 5min at 4°C. Discard supernatant.
 - Bring aliquot of PrimeFlow RNA wash buffer to room temperature.
10. Prepare **Fixation Buffer 1** by mixing equal parts of PrimeFlow RNA Fixation Buffer 1A and 1B (PFA based). Mix gently by inverting (no vortex/shaking). Prepare 1ml per sample.
11. Add 1ml of Fixation Buffer 1 to each sample and pipet to mix.
12. Incubate for 30min at 4°C.
13. Spin 800 x g 5min at 4°C. Discard supernatant.
14. Prepare **1x RNA Permeabilization Buffer with RNase Inhibitors**:
 - Dilute PrimeFlow RNA Permeabilization Buffer 10X to 1X with RNase-free water.
 - Add PrimeFlow RNase Inhibitors (100X) at 1/100 dilution.
 - Mix gently by pipet. Keep at 4°C. Prepare 3ml for each sample.
15. Wash twice in 1ml of 1x RNA Permeabilization Buffer with RNase Inhibitors, pipet to mix, and spin 800 x g 5min at 4°C. Discard supernatant.
16. Intracellular stain with FcR block followed by pS6-PE diluted in 1XPerm Buffer with RNase Inhibitors for 30min 4°C in the dark. Prepare single-stained controls.
17. Wash in 1ml of 1x RNA Permeabilization Buffer with RNase Inhibitors, pipet to mix, spin 800 x g 5min at 4°C. Discard supernatant. Transfer to PrimeFlow RNA tubes (ependorf size).
18. Prepare 1ml of **1x RNA Fixation Buffer 2** by combining 125µL of PrimeFlow RNA Fixation Buffer 2 (8X) with 875µL of PrimeFlow RNA Wash Buffer per sample. Mix gently by pipet.
19. Add 1ml of 1x RNA Fixation Buffer 2 to each sample and pipet to mix.
20. Incubate overnight in the dark at 4°C.

Overnight

- Set incubator with metal heat block to stably hold at 40 °C +/- 1°C (check by digital temperature probe), and set CO₂ very low (e.g. 0.2%) to avoid acidification.

Day 2: Target Probe Hybridization

- *Thaw PrimeFlow Target Probe Sets (20x) at room temperature.*
 - *Bring aliquot of PrimeFlow RNA wash buffer to room temperature.*
 - *Pre-warm PrimeFlow RNA Target Probe Diluent to 40°C.*
1. Spin samples *800 x g 5min* at room temperature. Aspirate all but 100µL of supernatant and resuspend cells in the residual volume by gentle vortex.
 2. Wash twice with 1ml of **PrimeFlow RNA Wash Buffer**, invert to mix, and spin at *800 x g 5min* at room temperature. Aspirate all but 100µL of supernatant and resuspend cells in the residual volume by gentle vortex.
 3. Dilute **Target Probes 1/20 in PrimeFlow RNA Target Probe Diluent**, prepare 100µl per sample. Mix thoroughly by pipetting up and down.
 4. Add 100µl diluted Target Probes directly into cell suspensions, briefly vortex to mix.
 5. Incubate 3hrs at 40°C. Invert samples to mix after 1.5hr.
 6. Wash by adding 1ml of **PrimeFlow RNA Wash Buffer** to each sample, invert to mix. Spin *800 x g 5min* at room temperature. Remove all but 100µl supernatant and gently vortex.
 7. Wash in 1ml of **PrimeFlow RNA Wash Buffer with RNase inhibitors** (1/100 dilution), invert to mix. Spin *800 x g 5min* at room temperature. Remove all but 100µl supernatant and gently vortex.

Signal Amplification

- *Pre-warm PrimeFlow RNA PreAmp Mix, PrimeFlow RNA Amp Mix, and PrimeFlow RNA Label Probe Diluent to 40°C.*
 - *Thaw PrimeFlow RNA Label Probes (100x) on ice in the dark (at point 10).*
8. Add 100µL of **PrimeFlow RNA PreAmp Mix** directly into the cell suspension for each sample and briefly vortex to mix.
 9. Incubate 2hrs at 40°C.
 10. Wash three times with 1ml of PrimeFlow RNA Wash Buffer, invert to mix. Spin *800 x g 5min* at room temperature. Remove all but 100µl supernatant and gently vortex.
 11. Add 100µL of **PrimeFlow RNA Amp Mix** directly into the cell suspension for each sample and briefly vortex to mix.
 12. Incubate 2hrs at 40°C.
 13. Wash twice in 1ml of PrimeFlow RNA Wash Buffer, invert to mix. Spin *800 x g 5min* at room temperature. Remove all but 100µl supernatant and gently vortex.
 14. Dilute **PrimeFlow RNA Label Probes (100X) 1:100 in PrimeFlow RNA Label Probe Diluent**.
 15. Add 100µL of **diluted Label Probes** directly into the cell suspension for each sample and briefly vortex to mix.
 16. Incubate 1.5 hr at 40°C.
 17. Wash twice in 1ml of PrimeFlow RNA Wash Buffer, invert to mix. Spin *800 x g 5min* at room temperature. Remove all but 100µl supernatant and gently vortex.
 18. Transfer to FACS tubes.
 19. Add 1ml of PrimeFlow RNA Storage Buffer or Flow Cytometry Staining Buffer to each sample, invert to mix. Spin *800 x g 5min* at room temperature. Discard supernatant.
 20. Resuspend in PrimeFlow RNA Storage Buffer or Flow Cytometry Staining Buffer and analyse on a flow cytometer.

Materials and Reagents:

1. CD8 T cell Isolation Kit, Mouse (MACS Miltenyi Biotec, catalogue number: 130-104-075)
2. MACS buffer: PBS with 0.5% BSA and 2 mM EDTA
3. Flow Cytometry Staining Buffer: PBS with 1% FBS
4. RPMI-1640 Medium (Merck, catalogue number: R8758)
5. Zombie Aqua Fixable Viability Kit (Biolegend, catalogue number: 423101)
6. Purified anti-mouse CD16/32 Antibody as FCR block (Biolegend, clone 93, catalogue number: 101335)
7. pS6 [S235/S236] - PE (BD Biosciences, clone N7-548, catalogue number: 560433)
8. PrimeFlow RNA Assay Kit (ThermoFisher, catalogue number: 88-18005-210)
9. PrimeFlow Target Probe - ribosomal protein L39 (Type 4/ AF488, ThermoFisher, catalogue number: VB4-3120826-204)
10. PrimeFlow Target Probe - Interferon regulatory factor 8 (Type 6/ AF750, ThermoFisher, catalogue number: VB6-3197312-210)
11. PrimeFlow Target Probe - nuclear receptor subfamily 4, group A, member 1 (Type 1/ AF647, ThermoFisher, catalogue number: VB1-12484-204)

How to cite: (Readers should cite both the Bio-protocol preprint and the original research article where this protocol was used)

1. Ma, C. , Marioni, J. , Richard, A. and Griffiths, G. (2021). Combined phosphoflow with RNA flow cytometry. Bio-protocol Preprint. bio-protocol.org/prep779.
2. Ma, C. Y., Marioni, J. C., Griffiths, G. M. and Richard, A. C. (2020). Stimulation strength controls the rate of initiation but not the molecular organisation of TCR-induced signalling. eLIFE. DOI: [10.7554/eLife.53948](https://doi.org/10.7554/eLife.53948)

Copyright: Content may be subjected to copyright.